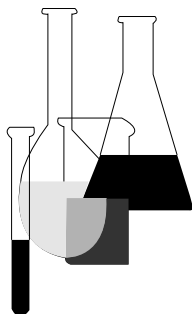




Health Effects Test Guidelines

OPPTS 870.3150 90-Day Oral Toxicity in Nonrodents



INTRODUCTION

This guideline is one of a series of test guidelines that have been developed by the Office of Prevention, Pesticides and Toxic Substances, United States Environmental Protection Agency for use in the testing of pesticides and toxic substances, and the development of test data that must be submitted to the Agency for review under Federal regulations.

The Office of Prevention, Pesticides and Toxic Substances (OPPTS) has developed this guideline through a process of harmonization that blended the testing guidance and requirements that existed in the Office of Pollution Prevention and Toxics (OPPT) and appeared in title 40, chapter I, subchapter R of the Code of Federal Regulations (CFR), the Office of Pesticide Programs (OPP) which appeared in publications of the National Technical Information Service (NTIS) and the guidelines published by the Organization for Economic Cooperation and Development (OECD).

The purpose of harmonizing these guidelines into a single set of OPPTS guidelines is to minimize variations among the testing procedures that must be performed to meet the data requirements of the U. S. Environmental Protection Agency under the Toxic Substances Control Act (15 U.S.C. 2601) and the Federal Insecticide, Fungicide and Rodenticide Act (7 U.S.C. 136, *et seq.*).

Final Guideline Release: This guideline is available from the U.S. Government Printing Office, Washington, DC 20402 on disks or paper copies: call (202) 512-0132. This guideline is also available electronically in PDF (portable document format) from EPA's World Wide Web site (<http://www.epa.gov/epahome/research.htm>) under the heading "Researchers and Scientists/Test Methods and Guidelines/OPPTS Harmonized Test Guidelines."

OPPTS 870.3150 90-day oral toxicity in nonrodents.

(a) **Scope—(1) Applicability.** This guideline is intended to meet testing requirements of both the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) (7 U.S.C. 136, *et seq.*) and the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) **Background.** The source materials used in developing this harmonized OPPTS test guideline is OPP 82-1 90-Day Oral—Two Species, Rodent and Nonrodent (Pesticide Assessment Guidelines, Subdivision F—Hazard Evaluation; Human and Domestic Animals) EPA report 540/09-82-025, 1982; and OECD 409 Subchronic Oral Toxicity—Nonrodent: 90-Day.

(b) **Purpose.** The determination of subchronic oral toxicity in the assessment and evaluation of the toxic characteristics of a chemical may be carried out after initial information on toxicity has been obtained by acute testing. The subchronic oral study has been designed to permit the determination of the no-observed-effect level (NOEL) and toxic effects associated with continuous or repeated exposure to a test substance for a period of 90 days. The test is not capable of determining those effects that have a long latency period for development (e.g. carcinogenicity and life shortening). Extrapolation from the results of this study to humans is valid only to a limited degree. However, it can be useful in providing information on health hazards likely to arise from repeated exposure by the oral route over a limited period of time. It provides information on target organs, the possibilities of accumulation, and can be of use in selecting dose levels for chronic studies and for establishing safety criteria for human exposure.

(c) **Definitions.** The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards (GLP) apply to this test guideline. The following definitions also apply to this test guideline.

Cumulative toxicity is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue.

Dose in an oral subchronic study is the amount of test substance administered daily via the oral route (gavage, capsules, diet or drinking water) for 90 days. Dose is expressed as weight of test substance (grams, milligrams) per unit weight of test animal (e.g. milligrams per kilogram), or as weight of test substance per unit weight of food or drinking water per day.

No-observed-effect-level (NOEL) is the maximum dose used in a test which produces no observed adverse effects. A NOEL is expressed in terms of the weight of a substance given daily per unit weight of test animal (milligrams per kilogram).

Subchronic oral toxicity is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral route for a part of the test animal's life span.

Target organ is any organ of a test animal showing evidence of an effect induced by the test substance.

(d) **Limit test.** If a test at one dose level of at least 1,000 mg/kg body weight (BW) (expected human exposure may indicate the need for a higher dose level), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, a full study using three dose levels might not be necessary.

(e) **Test procedures—(1) Animal selection—(i) Species and strain.** A mammalian nonrodent species should be used for testing. The commonly used nonrodent species is the dog, preferably of a defined breed; the beagle is frequently used. If other mammalian species are used, the tester should provide justification/reasoning for his or her selection.

(ii) **Age/weight.** (A) Young adult animals should be used.

(B) In the case of the dog, dosing should commence after acclimation, preferably at 4 to 6 months and not later than 9 months of age.

(C) At the commencement of the study the weight variation of animals used should be within 20 percent of the mean weight for each sex.

(iii) **Sex.** (A) Equal numbers of animals of each sex should be used at each dose level.

(B) The females should be nulliparous and nonpregnant.

(iv) **Numbers.** (A) At least eight animals (four females and four males) should be used at each dose level.

(B) If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the completion of the study.

(C) To avoid bias, the use of adequate randomization procedures for the proper allocation of animals to test and control groups is required.

(D) Each animal should be assigned a unique identification number. Dead animals, their preserved organs and tissues and microscopic slides should be identified by reference to the animal's unique number.

(v) **Husbandry.** (A) Caging and environmental conditions should be appropriate to the nonrodent species. However, it is recommended that dogs are housed individually. The number of animals per cage must not interfere with a clear observation of each animal.

(B) For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of the test substance when administered by this method.

(C) Control and test animals should be fed from the same batch and lot. The feed should be analyzed to assure adequacy of nutritional requirements of the species tested for impurities that might influence the outcome of the test. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

(D) The study should not be initiated until animals have been allowed a period of acclimatization/quarantine to environmental conditions, nor should animals from outside sources be placed on test without an adequate period of quarantine. An acclimation period of at least 5 days is recommended.

(2) **Control and test substances.** (i) Where necessary, the test substance is dissolved or suspended in a suitable vehicle. If a vehicle or diluent is needed, the vehicle should not elicit toxic effects or substantially alter the chemical or toxicological properties of the test substance. It is recommended that whenever possible the usage of an aqueous solution be considered first, followed by consideration of a solution of oil and then solution in other vehicles.

(ii) If possible, one lot of the test substance tested should be used throughout the duration of the study and the research sample should be stored under conditions that maintain its purity and stability. Prior to the initiation of the study, there should be characterization of the test substance, including the purity of the test compound and, if technically feasible, the names and quantities of contaminants and impurities.

(iii) If the test or control substance is to be incorporated into feed or another vehicle, the period during which the test substance is stable in such a mixture should be determined prior to the initiation of the study. Its homogeneity and concentration should be determined prior to the initiation of the study and periodically during the study. Statistically randomized samples of the mixture should be analyzed to ensure that proper mixing, formulation and storage procedures are being followed, and that the appropriate concentration of the test or control substance is contained in the mixture.

(3) **Control groups.** A concurrent control group is required. This group should be an untreated or sham-treated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the toxic properties of the vehicle are not known or cannot be made available, both untreated and vehicle control groups are required.

(4) **Satellite group.** A satellite group of eight animals (four animals per sex) may be treated with the high dose level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length, normally not less than 28 days. In addition, a control group of 8 animals (4 animals per sex) should be added to the satellite study.

(5) **Dose levels and dose selection.** (i) In subchronic toxicity tests, it is desirable to have a dose response relationship as well as a NOEL. Therefore, at least three dose levels with a control and, where appropriate, a vehicle control (corresponding to the concentration of vehicle at the highest exposure level) should be used. Doses should be spaced appropriately to produce test groups with a range of toxic effects. The data should be sufficient to produce a dose-response curve.

(ii) The highest dose level should result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation.

(iii) The intermediate dose levels should be spaced to produce a gradation of toxic effects.

(iv) The lowest dose level should not produce any evidence of toxicity.

(6) **Administration of the test substance.** (i) The test substance may be administered in the diet, drinking water, by gavage or in capsules. Ideally, if the test substance is administered by gavage or in capsules, the animals should be dosed with the test material on a 7-day per week basis for a period of at least 90 days. However, based primarily on practical considerations, dosing by gavage or with capsules on a 5-day per week basis is acceptable. If the test substance is administered in the drinking water or mixed in the diet, then exposure should be on a 7-day per week basis.

(ii) All animals should be dosed by the same method during the entire experimental period.

(iii) For substances of low toxicity, it is important to ensure that when administered in the diet the quantities of the test substance involved do not interfere with normal nutrition. When the test substance is administered in the diet, either a constant dietary concentration (parts per million) or a constant dose level in terms of the body weight of the animals should be used; the alternative used should be specified.

(iv) For a substance administered by gavage or capsules, the dose should be given at approximately the same time each day, and adjusted at intervals (weekly or biweekly) to maintain a constant dose level in terms of animal body weight.

(7) **Observation period.** (i) Duration of observation should be for at least 90 days.

(ii) Animals in the satellite group (if used) scheduled for follow-up observations should be kept for at least 28 days further without treatment to detect recovery from, or persistence of, toxic effects.

(8) **Observation of animals.** (i) Each animal should be observed twice daily for morbidity and mortality. Appropriate actions should be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead and isolation or sacrifice of weak or moribund animals.) General clinical observations should be made at least once a day, preferably at the same time each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animal should be recorded.

(ii) A careful clinical examination should be made prior to the initiation of treatment and at least once weekly during treatment. Detailed observations should be made on all animals. These observations should be made, where practical, outside the home cage in a standard arena and preferably at similar times on each occasion. Effort should be made to ensure that variations in the observation conditions are minimal. Signs of toxicity should be carefully recorded, including time of onset, degree and duration. Observations should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions, and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in level of activity, gait, posture, altered strength, and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilation) should be recorded.

(iii) Measurements of feed consumption and water consumption, when drinking water is the exposure route, should be made weekly.

(iv) Animals should be weighed shortly before the test substance is administered and weekly during the treatment period.

(v) Moribund animals should be removed and sacrificed when noticed and the time of death should be recorded as precisely as possible.

(vi) At the end of the 90-day period all survivors in the nonsatellite control and treatment groups should be sacrificed.

(9) **Clinical pathology.** Hematology and clinical chemistry examinations should be made on all animals, including controls, of each sex in each group. The hematology and clinical chemistry parameters should be examined prior to treatment, either at monthly intervals or midway through the treatment period, and at the end of the treatment period on all groups of animals, including concurrent controls. Overnight fasting of the animals

prior to blood sampling is recommended. Overall, there is a need for a flexible approach in the measures examined, depending on the observed or expected effects from a chemical, and in the frequency of measures, depending on the duration of potential chemical exposures.

(i) Hematology. The recommended parameters are red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration, white blood cell count, differential leukocyte count, platelet count, and a measure of clotting potential, such as prothrombin time and activated partial thromboplastin time.

(ii) Clinical chemistry. (A) Clinical biochemistry test areas which are considered appropriate to all studies are electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance and signs of clinical toxicity.

(B) The recommended clinical chemistry determinations are potassium, sodium, calcium, phosphorus, chloride, glucose, total cholesterol, urea nitrogen, creatinine, total protein, total bilirubin, and albumin. Suggested hepatic enzymes include alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, and gamma glutamyl transpeptidase. Measurements of additional enzymes (of hepatic or other origin) and bile acids may also be useful.

(C) If a test chemical has an effect on the hematopoietic system, reticulocyte counts and bone marrow cytology may be indicated.

(D) Other determinations that should be carried out if the test chemical is known or suspected of affecting related measures include fasting triglycerides, hormones, methemoglobin, and cholinesterases.

(iii) Urinalysis should be performed prior to treatment, midway through treatment and at the end of the study using timed urine collection. Urinalysis determinations include: appearance, volume, osmolality or specific gravity, pH, protein, glucose, and blood/blood cells.

(10) **Ophthalmological examination.** Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, should be made on all animals prior to the administration of the test substance and at termination of the study, preferably in all animals but at least the high dose and control groups. If changes in the eyes are detected, all animals in the other dose groups should be examined.

(11) **Gross necropsy.** (i) All animals should be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents.

(ii) At least the liver (with gall bladder), kidneys, adrenals, testes, epididymides, ovaries, uterus, thyroid (with parathyroid), thymus, spleen, brain, and heart should be weighed wet as soon as possible after dissection to avoid drying.

(iii) The following organs and tissues, or representative samples thereof, should be preserved in a suitable medium for possible future histopathological examination:

(A) Digestive system—salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, liver, pancreas, gallbladder.

(B) Nervous system—brain (multiple sections, including cerebrum, cerebellum and medulla/pons), pituitary, peripheral nerve (sciatic or tibia, preferably in close proximity to the muscle), spinal cord (three levels, cervical, mid-thoracic and lumbar), eyes (retina, optic nerve).

(C) Glandular system—adrenals, parathyroid, thyroid.

(D) Respiratory system—trachea, lungs, pharynx, larynx, nose.

(E) Cardiovascular/hematopoietic system—aorta, heart, bone marrow (and/or a fresh aspirate), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), spleen, thymus.

(F) Urogenital system—kidneys, urinary bladder, prostate, testes, epididymides, seminal vesicle(s), uterus, ovaries, female mammary gland.

(G) Other—all gross lesions and masses, skin.

(12) **Histopathology.** The following histopathology should be performed:

(i) Full histopathology on the organs and tissues, listed in paragraph (e)(11)(iii) of this guideline, in at least all animals in the control- and high-dose groups. The examination should be extended to all animals in all dosage groups if treatment-related changes are observed in the high-dose group.

(A) All gross lesions in all animals.

(B) Target organs in all animals.

(C) When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated group.

(ii) If excessive early deaths or other problems occur in the high dose group compromising the significance of the data, the next dose level should be examined for complete histopathology.

(iii) An attempt should be made to correlate gross observations with microscopic findings.

(iv) Tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming.

(f) **Data and reporting**—(1) **Treatment of results.** (i) Data should be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions and the percentage of animals displaying each type of lesion.

(ii) When applicable, all numerical results should be evaluated by an appropriate and generally acceptable statistical method. Any generally accepted statistical methods may be used; the statistical methods should be selected during the design of the study.

(2) **Evaluation of the study results.** (i) The findings of a subchronic oral toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the dose of the test substance and the presence or absence, the incidence and severity of abnormalities, including behavioral and clinical abnormalities, gross lesions, identified target organs, body weight changes, effects on mortality and any other general or specific toxic effects. A properly conducted subchronic test should provide a satisfactory estimation of a NOEL. It can also indicate the need for an additional longer-term study and provide information on selection of dose levels.

(3) **Test report.** In addition to the reporting requirements as specified under 40 CFR part 792, subpart J (Good Laboratory Practice Standards), 40 CFR part 160 and the OECD Principles of GLP (ISBN 92–64–12367–9) the following specific information should be reported:

(i) Test substance characterization should include:

(A) Chemical identification.

(B) Lot or batch numbers

(C) Physical properties.

(D) Purity/impurities.

(ii) Identification and composition of any vehicle used.

(iii) Test system should contain data on:

(A) Species and strain of animals used and rationale for selection if other than that recommended.

(B) Age, including body weight data and sex.

(C) Test environment including cage conditions, ambient temperature, humidity, and light/dark periods.

(D) Identification of animal diet.

(E) Acclimation period

(iv) Test procedure should include the following data:

(A) Method of randomization used.

(B) Full description of experimental design and procedure.

(C) Dose regime including levels, method, and volume.

(v) Test results should include:

(A) Group animal data. Tabulation of toxic response data by species, strain, sex, and exposure level for:

(1) Number of animals exposed.

(2) Number of animals showing signs of toxicity.

(3) Number of animals dying.

(B) Individual animal data. Data should be presented as summary (group mean) as well as for individual animals.

(1) Date of death during the study or whether animals survived to termination.

(2) Date of observation of each abnormal sign and its subsequent course.

(3) Body weight data.

(4) Feed and water (when collected) consumption data.

(5) Achieved dose (mg/kg/day) as a time-weighted average if the test substance is administered in the diet or drinking water.

(6) Ophthalmological examination data

(7) Hematological tests employed and all results.

(8) Clinical biochemistry tests employed and all results.

(9) Urinalysis tests employed and all results.

(10) Necropsy findings, including absolute and relative (to body weight) organ weight data.

(11) Detailed description of all histopathological findings.

(12) Statistical treatment of results, where appropriate.

(h) **Quality control.** A system should be developed and maintained to assure and document adequate performance of laboratory staff and equipment. The study must be conducted in compliance with GLP regulations.

(i) **References.** The following references should be consulted for additional background information on this test guideline.

(1) Boyd, E.M. Chapter 14—Pilot Studies, 15—Uniposal Clinical Parameters, 16—Uniposal Autopsy Parameters, in *Predictive Toxicometrics*. Williams and Wilkins, Baltimore, MD (1972).

(2) Fitzhugh, O.G. Subacute Toxicity, in *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*. The Association of Food and Drug Officials of the United States (1959, 3rd Printing 1975) pp. 26–35.

(3) Food Safety Council. Subchronic Toxicity Studies, in *Proposed System for Food Safety Assessment*. Food Safety Council, Columbia (1978) pp. 83–96.

(4) National Academy of Sciences. Principles and Procedures for Evaluating the Toxicity of Household Substances, a report prepared by the Committee for the Revision of NAS Publication 1138, under the auspices of the Committee on Toxicology, National Research Council, National Academy of Sciences, Washington, DC (1977).

(5) Weingand K., Brown G., Hall R. et al. Harmonization of Animal Clinical Pathology Testing in Toxicity and Safety Studies. *Fundam. & Appl. Toxicol.* 29:198–201 (1996).

(6) World Health Organization. Part I. Environmental Health Criteria 6, in *Principles and Methods for Evaluating the Toxicity of Chemicals*. World Health Organization, Geneva (1978).